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Erhvervsministeriet

TAASTRUP 01 Dec 1999

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A TRANSGENIC PLANT EXPRESSING A NOVAMYL-LIKE AMYLASE

FIELD OF THE INVENTION

The present invention relates to a cell of a transgenic seed-producing plant expressing a Novamyl-like amylase, a transgenic plant regenerated from said cell, seeds comprising a measurable quantity of a Novamyl-like amylase and the use of said seeds, optionally in ground form, for catalyzing an industrial process.

BACKGROUND OF THE INVENTION

Novamyl® (Novo Nordisk A/S, Denmark) is a commercially important enzyme which has been classified as a maltogenic amylase (glucan 1,4-a-maltohydrolase, E.C. 3.2.1.133) and which is widely used in the baking industry as an antistaling agent due to its ability to reduce retrogradation of amylopectin. Novamyl is further described by Christophersen, C., et al., 1998, Starch 50, pp 39-45.

WO 91/14772 discloses transgenic seeds expressing enhanced amounts of enzymes, and the use of such seeds in catalyzing industrial processes. Baking is mentioned as one example of an industrial process for which α-amylase can be used and it is stated that the seeds may be ground before being incorporated into flour.

Vickers et al, Journal of the Institute of Brewing, Vol. 102, No. 2 pp. 75-78 (1996) speculate in using a *Bacillus licheniformis* α-amylase as a candidate enzyme for the genetic transformation of malting barley.

BRIEF DESCRIPTION OF THE INVENTION

The present invention relates to a cell of a transgenic seed producing plant transformed with at least one nucleotide sequence encoding a "Novamyl-like" amylase which, in the cell, is operably linked to elements required for mediating expression

from said nucleotide sequence in the seeds of a plant regenerated from the plant cell.

In a further aspect the invention relates to a transgenic seed-producing plant regenerated from a cell of the invention and expressing measurable quantities of a Novamyl-like amylase in its seeds.

In a still further aspect the invention relates to the seeds of a plant of the invention, optionally in ground form, and the use of such seed for catalyzing an industrial process.

In a final aspect the invention relates to a method for producing a Novamyl-like amylase comprising recovering the amylase from seeds of the invention.

DETAILED DISCLOSURE OF THE INVENTION

In the present context the term "transgenic plant" is intended to mean a plant which has been genetically modified to express a Novamyl-like amylase and progeny of such plant having retained the capability of producing a Novamyl-like amylase. The term also includes a part of such plant such as a leave, seed, stem, any tissue from the plant, an organelle, a cell of the plant, etc.

In the present context the term "Novamyl-like amylase" is intended to mean an enzyme with one or more characteristics selected from the group consisting of:

i) an amino acid sequence similar to the amino acid sequence set forth in SEQ ID NO:2;

ii) a DNA sequence which hybridizes to the DNA sequence set forth in SEQ ID NO:1 or to the DNA sequence encoding Novamyl harboured in the *Bacillus* strain NCIB 11837; and

iii) a catalytic binding site comprising amino acid residues similar to D229, E257 and D328 as shown in the amino acid sequence set forth in SEQ ID NO:2.

The polypeptide sequence similarity referred to above in i) is determined as the degree of homology between two sequences indicating a derivation of the first sequence from the second. The similarity may be suitably determined by means of computer programs known in the art such as GAP, provided in the GCG program package (Program Manual for the Wisconsin Package,

Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711; Needleman, S.B. and Wunsch, C.D., 1970, Journal of Molecular Biology, 48, 443-453). Using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1, the mature protein part of a polypeptide encoded by an analogous DNA sequence of the invention exhibits a degree of similarity preferably of at least 40%, preferably at least 50%, more preferably at least 60%, most preferably at least 67% amino acid similarity to the amino acid sequence set forth in SEQ ID NO:2.

In connection with Novamyl-like amylases characterized by ii), the oligonucleotide probe used in a hybridization may be suitably prepared on the basis of the nucleic acid sequence set forth in SEQ ID NO:1.

The hybridization referred to above in ii) is intended to indicate that the analogous DNA sequence hybridizes to the nucleotide probe corresponding to the protein encoding part of the nucleic sequence shown in SEQ ID NO:1, under at least low stringency conditions as described in detail below.

Suitable experimental conditions for determining hybridization at low stringency between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5x SSC (sodium chloride/sodium citrate, Sambrook, et al., 1989) for 10 min, and prehybridization of the filter in a solution of 5x SSC, 5x Denhardt's solution (Sambrook, et al., 1989), 0.5% SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook, et al., 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed twice for 30 minutes in 2x SSC, 0.5% SDS at least 55°C (low stringency), more preferably at least 60°C (medium stringency), more preferably at least 65°C (medium/high stringency), more preferably at least 70°C (high stringency), even more preferably at least 75°C (very high stringency).

Molecules which hybridize to the oligonucleotide probe under these conditions are detected by exposure to x-ray film.

Cloning a DNA sequence encoding a Novamyl-like amylase

The nucleotide sequence encoding the Novamyl-like amylase may be of any origin, including mammalian, plant and microbial origin and may be isolated from these sources by conventional methods. Preferably, the nucleotide sequence is derived from a microorganism, such as a fungus, eg a yeast or a filamentous fungus, or a bacterium. The DNA sequence encoding a parent Novamyl-like amylase may be isolated from the cell producing the Novamyl-like amylase in question, using various methods well known in the art, for example, from the *Bacillus* strain NCIB 11837.

First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the Novamyl-like amylase to be studied. Then, if the amino acid sequence of the Novamyl-like amylase is known, homologous, labelled oligonucleotide probes may be synthesised and used to identify Novamyl-like amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known Novamyl-like amylase gene could be used as a probe to identify Novamyl-like amylase-encoding clones, using hybridization and washing conditions of lower stringency.

Another method for identifying Novamyl-like amylase-encoding clones involves inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming Novamyl-like amylase negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for Novamyl-like amylase, thereby allowing clones expressing Novamyl-like amylase activity to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by S.L. Beaucage and M.H. Caruthers (1981) or the method described by Matthes et al. (1984). In the phosphoroamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or

Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711; Needleman, S.B. and Wunsch, C.D., 1970, Journal of Molecular Biology, 48, 443-453). Using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1, the mature protein part of a polypeptide encoded by an analogous DNA sequence of the invention exhibits a degree of similarity preferably of at least 40%, preferably at least 50%, more preferably at least 60%, most preferably at least 67% amino acid similarity to the amino acid sequence set forth in SEQ ID NO:2.

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First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the Novamyl-like amylase to be studied. Then, if the amino acid sequence of the Novamyl-like amylase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify Novamyl-like amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known Novamyl-like amylase gene could be used as a probe to identify Novamyl-like amylase-encoding clones, using hybridization and washing conditions of lower stringency.

Another method for identifying Novamyl-like amylase-encoding clones involves inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming Novamyl-like amylase negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for Novamyl-like amylase, thereby allowing clones expressing Novamyl-like amylase activity to be identified.

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Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or

cDNA origin, wherein the fragments correspond to various parts of the entire DNA sequence, in accordance with techniques well known in the art. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988).

Expression Constructs

In order to accomplish expression of the Novamyl-like amylase in seeds of the transgenic plant of the invention the nucleotide sequence encoding the amylase is inserted into an expression construct containing regulatory elements capable of directing the expression of the nucleotide sequence and, if necessary, to direct secretion of the gene product or targetting of the gene product to the seeds of the plant. Manipulation of nucleotide sequences using restriction endonucleases to cleave DNA molecules into fragments and DNA ligase enzymes to unite compatible fragments into a single DNA molecule with subsequent incorporation into a suitable plasmid, cosmid, or other transformation vector are well-known in the art.

In order for transcription to occur the nucleotide sequence encoding the Novamyl-like amylase is operably linked to a suitable promoter capable of mediating transcription in the plant in question. The promoter may be an inducible promoter or a constitutive promoter. Typically, an inducible promoter mediates transcription in a tissue-specific or growth-stage specific manner, whereas a constitutive promoter provide for sustained transcription in all cell tissues. As examples of a suitable constitutive promoter useful for the present invention the cauliflower mosaic virus 35 S promoter. Other constitutive promoters are a transcription initiation sequence from the tumor-inducing plasmid (Ti) of *Agrobacterium* such as the octopine synthase, nopaline synthase, or mannopine synthase initiator.

Examples of suitable inducible promoters include a seed-specific promoter such as the promoter expressing α-amylase in wheat seeds (see Stefanov et al, Acta Biologica Hungarica Vol. 42, No. 4 pp. 323-330 (1991), a promoter of the gene encoding a rice seed storage protein such as glutelin, prolamin, globulin

or albumin (Wu et al., Plant and Cell Physiology Vol. 39, No. 8 pp. 885-889 (1998)), a *Vicia faba* promoter from the legumin B4 and the unknown seed protein gene from *Vicia faba* described by Conrad U. et al, Journal of Plant Physiology Vol. 152, No. 6 pp. 708-711 (1998), the storage protein napA promoter from Brassica napus, or any other seed specific promoter known in the art, eg as described in WO 91/14772.

In order to increase the expression of the Novamyl-like amylase it is desirable that a promoter enhancer element is used. For instance, the promoter enhancer may be an intron which is placed between the promoter and the amylase gene. The intron may be one derived from a monocot or a dicot. For instance, the intron may be the first intron from the rice Waxy (Wx) gene (Li et al., Plant Science Vol. 108, No. 2, pp. 181-190 (1995)), the first intron from the maize Ubi1 (Ubiquitin) gene (Vain et al., Plant Cell Reports Vol. 15, No. 7 pp. 489-494 (1996)) or the first intron from the Act1 (actin) gene. As an example of a dicot intron the chsA intron (Vain et al. op cit.) is mentioned. Also, a seed specific enhancer may be used for increase the expression of the Novamyl-like amylase in seeds. An example of a seed specific enhancer is the one derived from the beta-phaseolin gene encoding the major seed storage protein of bean (Phaseolus vulgaris) disclosed by Vandergeest and Hall, Plant Molecular Biology Vol. 32, No. 4, pp. 579-588 (1996).

Also, the expression construct contains a terminator sequence to signal transcription termination of the Novamyl-like amylase gene such as the rbcS2' and the nos3' terminators.

To facilitate selection of successfully transformed plants, the expression construct should also include one or more selectable markers, eg an antibiotic resistance selection marker or a selection marker providing resistance to a herbicide. One widely used selection marker is the neomycin phosphotransferase gene (NPTII) which provides kanamycin resistance. Examples of other suitable markers include a marker providing a measurable enzyme activity, eg dihydrofolate reductase, luciferase, and β-glucoronidase (GUS). Phosphinothricin acetyl transferase may be used as a selection marker in combination with the herbicide basta or bialaphos.

Transgenic plant species

Any transformable seed-producing plant species may be used for the present invention. Of particular interest is a monocotyledonous plant species, in particular crop or cereal plants such as wheat (*Triticum*, eg *aestivum*), barley (*Hardeum*, eg *vulgare*), oats, rye, rice, sorghum and corn (*Zea*, eg *mays*). In particular, wheat is preferred.

Transformation of plants

The transgenic plant cell of the invention may be prepared by methods known in the art. The transformation method used will depend on the plant species to be transformed and can be selected from any of the transformation methods known in the art such as *Agrobacterium* mediated transformation (Zambryski et al., EMBO Journal 2, pp 2143-2150, 1993), particle bombardment, electroporation (Fromm et al. 1986, Nature 319, pp 791-793), and virus mediated transformation. For transformation of monocots particle bombardment (ie biolistic transformation) of embryogenic cell lines or cultured embryos are preferred. In the following references disclosing methods for transforming different plants are mentioned together with the plant: Rice (Cristou et al. 1991, Bio/Technology 9, pp. 957-962), Maize (Gordon-Kamm et al. 1990, Plant Cell 2, pp. 603-618), Oat (Somers et al. 1992, Bio/Technology 10, pp 1589-1594), Wheat (Vasil et al. 1991, Bio/Technology 10, pp. 667-674, Weeks et al. 1993, Plant Physiology 102, pp. 1077-1084) and barley (Wan and Lemaux 1994, Plant Physiology 102, pp. 37-48, review Vasil 1994, Plant Mol. Biol. 25, pp 925-937).

More specifically, *Agrobacterium* mediated transformation is conveniently achieved as follows:
A vector system carrying the Novamyl-like amylase is constructed. The vector system may comprise of one vector, but it can comprise of two vectors. In the case of two vectors the vector system is referred to as a binary vector system (Gynheung An et al.(1980), Binary Vectors, Plant Molecular Biology Manual A3, 1-19).

An *Agrobacterium* based plant transformation vector consists of replication origin(s) for both *E.coli* and *Agrobacterium* and a bacterial selection marker. A right and preferably also a left border from the Ti plasmid from *Agrobacterium tumefaciens* or from the Ri plasmid from *Agrobacterium rhizogens* is nessesary for the transformation of the plant. Between the borders the expression construct is placed which contains the Novamyl-like amylase gene and appropriate regulatory sequences such as promotor and terminator sequences. Additionally, a selection gene eg the neomycin phosphotransferase type II (NPTII) gene from transposon Tn5 and a reporter gene such as the GUS (betha-glucuronidase) gene is cloned between the borders. A disarmed Agrobacterium strain harboring a helper plasmid containing the virulens genes is transformed with the above vector. The transformed Agrobacterium strain is then used for plant transformation.

Industrial processes

In principle, the seeds of the invention may be used in any industrial process for which purified Novamyl-like amylases are normally used to catalyze a reaction between one or more substrate so as to produce the desired effects or products. Of particular interest for the present invention is the use of the seeds in the bread making process for improving the properties of a dough or a baked product. According to one embodiment of the present invention the seeds of the invention are used directly in the baking process without the need for first extracting and/or isolating the enzyme. For use in a baking process it is preferred that the seeds containing the Novamyl-like amylase are milled so as to obtain a consistency suitable for baking.

According to one aspect of the invention the seeds, optionally in a ground form, are used for preparing a flour, in particular wheat flour. More specifically, the flour may be prepared by milling seeds of the invention containing a Novamyl-like amylase. The milling may be conducted in accordance with methods known in the art for preparing flour from seeds.

When a flour has been produced from seeds of the present invention the Novamyl-like amylase activity of the resulting flour is normally measured and the strength of the enzyme activity adjusted. For instance, if too much Novamyl-like amylase activity is present in the flour prepared from transgenic seeds of the invention the flour may be diluted with flour free from the Novamyl-like amylase. If too little Novamyl-like amylase activity is present in the flour additional activity may be added, eg in the form of an isolated Novamyl-like amylase, such as Novamyl® available from Novo Nordisk A/S. It follows, that the flour of the present invention may be prepared exclusively from transgenic seeds containing a Novamyl-like amylase or from a mixture of seeds which in addition to the transgenic seed of the invention contains non-transgenic seeds or seeds, which otherwise do not contain the Novamyl-like amylase.

The flour of the invention is used in accordance with conventional techniques for the production of baked products, in particular bread products. The resulting baked product has an improved antistaling effect, ie the baked product has a reduced rate of deterioration of quality parameters, *e.g.*, softness and/or elasticity, during storage.

The Novamyl-like amylase has a very unique performance in bread making. Other thermostable α-amylases like BAN® or Termamyl® must be dosed very carefully in tight intervals, e.g. between 0.5-2 times of the optimum dosage in a given recipe. Otherwise the risk is high that there is either no effect (low dosage) or too high effect (high dosage). The latter will result in a gummy, non-elastic and sticky crumb, unsuited for eating. The Novamyl-like amylases as represented by Novamyl® does not have this problem, but can be dosed broadly. For instance, Novamyl® has a positive function on e.g. staling properties from a level of e.g. 200 MANU/kg flour to 5.000 MANU/kg, i.e. a much safer amylase in practical application than other α-amylases.

The term "baked product" is defined herein as any product prepared from a dough, either of a soft or a crisp character. Examples of baked products, whether of a white, light or dark type, which may be advantageously produced by the present

invention are bread (in particular white, whole-meal or rye bread), typically in the form of loaves or rolls, French baguette-type bread, pasta, pita bread, tortillas, tacos, cakes, pancakes, biscuits, cookies, pie crusts, steamed bread, and crisp bread, and the like.

In terms of enzyme activity, the appropriate dosage of the Novamyl-like amylase for exerting a desirable improvement of dough and/or baked products, in particular improved anti-staling properties, will depend on the specific amylase and the amylase substrate in question. The skilled person may determine a suitable enzyme unit dosage on the basis of methods known in the art. Normally, a suitable dosage of the Novamyl-like amylase (as present in the flour) is in the range 200 - 5.000 MANU/kg flour.

EXAMPLES

MATERIALS AND METHODS

Transformation of Wheat

Wheat plants are grown in greenhouse and caryopses are isolated and surface sterilised with hypochlorite. Immature embryos are dissected from the young caryopses and transferred to callus maintenance medium. Gold particles are coated with the vector plasmid containing the Novamyl-like amylase gene and regulatory sequences together with the selection marker phosphinothricin acetyl transferase providing herbicidal resistance. The embryo-derived calli or immature embryos are bombarded and immediately after transferred to selection medium containing 1mg/l Basta or Bialaphos. Resistant calli are transferred to regeneration medium in light. Green shoots with root system are transferred to soil.

Example 1

The gene encoding Novamyl (SEQ ID No 1) is operably linked to the wheat promoter expressing α-amylase in wheat seeds as described in "Promoter and genotype dependent transient

expression of a reporter gene in plant protoplasts."; Stefanov I ; Ilubaev S ; Feher A ; Margoczi K ; Dudits D; Acta Biologica Hungarica Vol. 42 , No. 4 pp. 323-330 (1991 The resulting DNA construct is inserted into a plasmid containing suitable regulatory elements and a selection marker.

Protoplasts are isolated from wheat cell lines as described in ("Culture of and fertile plant-regeneration from regenerable embryogenic suspension cell-derived protoplasts of wheat(triticum-aestivum l)"; Ahmed, KZ ; Sagi, F; PLANT CELL REPORTS Vol. 12 , pp. 175-179 (1993).

The DNA construct containing the Novamyl-like amylase is inserted into wheat protoplast cells via PEG treatment as described in "Factors affecting transient expression of vector constructs in wheat protoplasts."; Ahmed KZ ; Omirulleh S ; Sagi F ; Dudits D; Acta Biologica Hungarica Vol. 48 , No. 2 pp. 209-220 (1997).

The resulting protoplast is regenerated into a wheat plant as described in "Fertile wheat (Triticum aestivum L.) regenerants from protoplasts of embryogenic suspension culture."; Pauk J ; Kertesz Z ; Jenes B ; Purnhauser L ; Manninen O ; Pulli S ; Barbas Z ; Dudits D; Plant Cell Tissue and Organ Culture Vol. 38, No. 1 pp. 1-10 (1994).

The seeds are harvested, and multiplied and used for producing transgenic wheat plant expressing Novamyl in its seeds.

Example 2

The wheat seeds are milled in accordance with conventional techniques for the preparation of wheat flour. Optionally, the wheat is allowed to malt to a predetermined degree before milling. This will allow a greater expression of the bacterial enzyme. The Novamyl content of the flour is determined in MANU: One MANU (Maltogenic Amylase Novo Unit) is defined as the amount of enzyme required to release one mmol of maltose per minute at a concentration of 10 mg of maltotriose (Sigma M 8378) substrate per ml of 0.1 M citrate buffer, pH 5.0 at 37 °C for 30 minutes.

If needed the content of Novamyl in the flour is adjusted as discussed above in the Detailed Description so as to result

in a Novamyl content pr kg of flour in the range of 200-5000 MANU/kg of flour.

Example 3

A baking trial is carried out. The transformed flour is compared to the original un-transformed wheat "sort". The optimum water absorption is determined on a Farinograph (AACC method The Farinograph Handbook, 3rd Edition, 1984, AACC, Edited by Bert L. D'Appolonia and Wallace H. Kunerth, ISBN 0-913250-37-6).

Preparation of White Bread (I)

The straight-dough bread-making method may be used according to AACC Method 10-10B (in Approved Methods of the American Association of Cereal Chemists, Ninth Edition, March 1995; AACC, St. Paul MN, USA).

Basic recipe

Wheat flour	100%
Salt	1.5%
Yeast (fresh)	5.3%
Sugar	6.0%
Shortening	3.0%
Water	optimum

All percentages are by weight relative to the wheat flour.

Procedure

1. Dough mixing (Hobart mixer):
   The mixing time and speed should be determined by the skilled baker so as to obtain an optimum dough consistency under the testing conditions used.
2. 1st punch (e.g., 52 minutes after start)
3. 2nd punch (e.g., 25 minutes later)
4. Molding and panning (e.g., 13 minutes later).
5. Proofing to desired height (e.g., 33 minutes at 32°C, 82% RH)
5. Baking (e.g., at 215°C for 24 minutes)

Preparation of White Bread (II)

The sponge-dough bread-making method may be used according to AACC Method 10-11 (in Approved Methods of the American Association of Cereal Chemists, Ninth Edition, March 1995; AACC, St. Paul MN, USA).

Basic recipe for Sponge

Wheat flour	60%
Yeast (compressed)	36%
Yeast Food	2%
Water	36%

All percentages are by weight relative to the wheat flour.

Procedure

1. Add water to compressed yeast
2. Add yeast food in dry form with flour
3. Mix sponge (Hobart A-120; Hobart Corp., Troy OH, USA):
   0.5 minute at 1st speed
   1 minute at 2nd speed
   The mixing time may be adjusted so as to obtain an optimum dough consistency under the testing conditions used.
4. Ferment in a fermentation cabinet: 4 hours at 30°C, 85% RH

Basic recipe for Dough

Wheat flour	40%
Water	24%
Sugar	5%
Shortening	3%
Salt	2%

All percentages are by weight relative to the wheat flour.

Procedure

1. Add dough ingredients; begin mixer (1st speed)
2. Add sponge in three approximately equal portions at 15, 25, and 35 seconds mixing time; total mixing time: 1 minute
3. At 2nd speed, mix to obtain an optimum dough consistency
4. Ferment in a fermentation cabinet: 30 minutes at 30°C, 85% RH
5. Intermediate proof: 12-15 minutes in fermentation cabinet
6. Mold and final proof at 35.5°C, 92% RH
7. Bake: 25 minutes at 218°C

Example 4

Evaluation of Staling Properties of Bread

The degree of staling is determined on bread, *e.g.*, on day 1, 3, 7 and 9 after baking. Evaluation of staleness and texture can be done according to AACC method 74-09. The principles for determination of softness and elasticity of bread crumb are as follows:

1. A slice of bread is compressed with a constant speed in a texture analyser, measuring the force for compression in g.
2. The softness of the crumb is measured as the force at 25% compression.
3. The force at 40% compression (P2) and after keeping 40% compression constant for 30 seconds (P3) is measured. The ratio (P3/P2) is the elasticity of the crumb.

CLAIMS

1. A transgenic seed producing plant cell transformed with at least one nucleotide sequence encoding a "Novamyl-like" amylase which, in the cell, is operably linked to elements required for mediating expression from said nucleotide sequence in the seeds of a plant regenerated from the plant cell.

2. The plant cell according to claim 1, wherein the Novamyl-like amylase has the amino acid sequence shown in SEQ ID NO 2.

3. The plant cell according to claim 2, wherein the nucleotide sequence encoding the Novamyl-like amylase is derived from a microorganism.

4. The plant cell according to claim 3, wherein the nucleotide sequence encoding the Novamyl-like amylase is derived from the *Bacillus* strain NCIB 11837.

5. The plant cell according to claim 1, wherein the promoter driving the expression of the Novamyl-like amylase is a seed-specific promoter.

6. The plant cell according to any of the preceding claims, wherein the plant is a monocotyledoneous plant.

7. The plant cell according to claim 6, which is wheat.

8. The plant cell according to any of the preceding claims wherein the plant is wheat and the Novamyl-like amylase is encoded by the DNA sequence shown in SEQ ID NO 1.

9. A transgenic seed producing plant regenerated from a plant cell according to any of the preceding claims and the progeny of said plant.

10. The plant according to claim 9 which is wheat.

11. A seed of a plant according to any of claims 9 or 10 containing a measurable amount of a Novamyl-like amylase.

12. A DNA construct comprising a nucleotide sequence encoding a Novamyl-like amylase and one or more regulatory elements capable of directing the expression of the nucleotide sequence and if necessary to direct secretion of the gene product to the seeds of the plant.

13. A method of producing a Novamyl-like amylase, which method comprises recovering the amylase from a seed according to claim 11.

14. A ground seed preparation containing a Novamyl-like amylase prepared by grinding a seed according to claim 11.

15. Flour prepared from a ground seed preparation according to claim 14.

16. Use of a seed according to claim 11 or a seed preparation according to claim 14 for catalyzing an industrial process.

The use according to claim 16, wherein the industrial process is baking.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): 2880
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(ii) TITLE OF INVENTION: TITLE

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2150 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(B) STRAIN: Bacillus sp NCIB 11839

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGAAAAAGA AAACGCTTTC TTTATTTGTG GGACTGATGC TCCTCATCGG TCTTCTGTTC 60

AGCGGTTCTC TTCCGTACAA TCCAAACGCC GCTGAAGCCA GCAGTTCCGC AAGCGTCAAA 120

GGGGACGTGA TTTACCAGAT TATCATTGAC CGGTTTTACG ATGGGGACAC GACGAACAAC 180

AATCCTGCCA AAAGTTATGG ACTTTACGAT CCGACCAAAT CGAAGTGGAA AATGTATTGG 240

GGCGGGGATC TGGAGGGGGT TCGTCAAAAA CTTCCTTATC TTAAACAGCT GGGCGTAACG 300

ACAATCTGGT TGTCCCCGGT TTTGGACAAT CTGGATACAC TGGCGGGCAC CGATAACACG 360

GGCTATCACG GATACTGGAC GCGCGATTTT AAACAGATTG AGGAACATTT CGGGAATTGG 420

ACCACATTTG ACACGTTGGT CAATGATGCT CACCAAAACG GAATCAAGGT GATTGTCGAC 480

TTTGTGCCCA ATCATTCGAC TCCTTTTAAG GCAAACGATT CCACCTTTGC GGAAGGCGGC 540

GCCCTCTACA ACAATGGAAC CTATATGGGC AATTATTTTG ATGACGCAAC AAAAGGGTAC 600

TTCCACCATA ATGGGGACAT CAGCAACTGG GACGACCGGT ACGAGGCGCA ATGGAAAAAC 660

TTCACGGATC CAGCCGGTTT CTCGCTTGCC GATTTGTCGC AGGAAAATGG CACGATTGCT 720

CAATACCTGA CCGATGCGGC GGTTCAATTG GTAGCACATG GAGCGGATGG TTTGCGGATT 780

GATGCGGTGA AGCATTTTAA TTCGGGGTTC TCCAAATCGT TGGCCGATAA ACTGTACCAA 840

AAGAAAGACA TTTTCCTGGT GGGGGAATGG TACGGAGATG ACCCCGGAAC AGCCAATCAT 900

CTGGAAAAGG TCCGGTACGC CAACAACAGC GGTGTCAATG TGCTGGATTT TGATCTCAAC 960

ACGGTGATTC GAAATGTGTT CGGCACATTT ACGCAAACGA TGTACGATCT TAACAATATG 1020

GTGAACCAAA CGGGGAACGA GTACAAATAC AAAGAAAATC TAATCACATT TATCGATAAC 1080

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CATGATATGT CAAGATTTCT TTCGGTAAAT TCGAACAAGG CGAATTTGCA CCAGGCGCTT 1140

GCTTTCATTC TCACTTCGCG GGGTACGCCC TCCATCTATT ATGGAACCGA ACAATACATG 1200

GCAGGCGGCA ATGACCCGTA CAACCGGGGG ATGATGCCGG CGTTTGATAC GACAACCACC 1260

GCCTTTAAAG AGGTGTCAAC TCTGGCGGGG TTGCGCAGGA ACAATGCGGC GATCCAGTAC 1320

GGCACCACCA CCCAGCGTTG GATCAACAAT GATGTTTACA TTTATGAACG GAAATTTTTC 1380

AACGATGTCG TGTTGGTGGC CATCAATCGA AACACGCAAT CCTCCTATTC GATTTCCGGT 1440

TTGCAGACGG CCTTGCCAAA TGGCAGCTAT GCGGATTATC TGTCAGGGCT GTTGGGGGGG 1500

AACGGGATTT CCGTTTCCAA TGGAAGTGTC GCTTCGTTCA CGCTTGCGCC TGGAGCCGTG 1560

TCTGTTTGGC AGTACAGCAC ATCCGCTTCA GCGCCGCAAA TCGGATCGGT TGCTCCAAAT 1620

ATGGGGATTC CGGGTAATGT GGTCACGATC GACGGGAAAG GTTTTGGGAC GACGCAGGGA 1680

ACCGTGACAT TTGGCGGAGT GACAGCGACT GTGAAATCCT GGACATCCAA TCGGATTGAA 1740

GTGTACGTTC CCAACATGGC CGCCGGGCTG ACCGATGTGA AAGTCACCGC GGGTGGAGTT 1800

TCCAGCAATC TGTATTCTTA CAATATTTTG AGTGGAACGC AGACATCGGT TGTGTTTACT 1860

GTGAAAAGTG CGCCTCCGAC CAACCTGGGG GATAAGATTT ACCTGACGGG CAACATACCG 1920

GAATTGGGGA ATTGGAGCAC GGATACGAGC GGAGCCGTTA ACAATGCGCA AGGGCCCCTG 1980

CTCGCGCCCA ATTATCCGGA TTGGTTTTAT GTATTCAGCG TTCCAGCAGG AAAGACGATT 2040

CAATTCAAGT TCTTCATCAA GCGTGCGGAT GGAACGATTC AATGGGAGAA TGGTTCGAAC 2100

CACGTGGCCA CAACTCCCAC GGGTGCAACC GGTAACATTA CTGTTACGTG 2150

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 686 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ser Ser Ser Ala Ser Val Lys Gly Asp Val Ile Tyr Gln Ile Ile Ile
1 5 10 15

Asp Arg Phe Tyr Asp Gly Asp Thr Thr Asn Asn Asn Pro Ala Lys Ser
20 25 30

Tyr Gly Leu Tyr Asp Pro Thr Lys Ser Lys Trp Lys Met Tyr Trp Gly
35 40 45

Gly Asp Leu Glu Gly Val Arg Gln Lys Leu Pro Tyr Leu Lys Gln Leu
50 55 60

Gly Val Thr Thr Ile Trp Leu Ser Pro Val Leu Asp Asn Leu Asp Thr
65 70 75 80

Leu Ala Gly Thr Asp Asn Thr Gly Tyr His Gly Tyr Trp Thr Arg Asp
85 90 95

Phe Lys Gln Ile Glu Glu His Phe Gly Asn Trp Thr Thr Phe Asp Thr
100 105 110

Leu Val Asn Asp Ala His Gln Asn Gly Ile Lys Val Ile Val Asp Phe
115 120 125

Val Pro Asn His Ser Thr Pro Phe Lys Ala Asn Asp Ser Thr Phe Ala
130 135 140

Glu Gly Gly Ala Leu Tyr Asn Asn Gly Thr Tyr Met Gly Asn Tyr Phe
145 150 155 160

Asp Asp Ala Thr Lys Gly Tyr Phe His His Asn Gly Asp Ile Ser Asn
165 170 175

Trp Asp Asp Arg Tyr Glu Ala Gln Trp Lys Asn Phe Thr Asp Pro Ala
180 185 190

Gly Phe Ser Leu Ala Asp Leu Ser Gln Glu Asn Gly Thr Ile Ala Gln
195 200 205

Tyr Leu Thr Asp Ala Ala Val Gln Leu Val Ala His Gly Ala Asp Gly
210 215 220

Leu Arg Ile Asp Ala Val Lys His Phe Asn Ser Gly Phe Ser Lys Ser
225 230 235 240

Leu Ala Asp Lys Leu Tyr Gln Lys Lys Asp Ile Phe Leu Val Gly Glu
245 250 255

Trp Tyr Gly Asp Asp Pro Gly Thr Ala Asn His Leu Glu Lys Val Arg
260 265 270

Tyr Ala Asn Asn Ser Gly Val Asn Val Leu Asp Phe Asp Leu Asn Thr
275 280 285

Val Ile Arg Asn Val Phe Gly Thr Phe Thr Gln Thr Met Tyr Asp Leu
290 295 300

Asn Asn Met Val Asn Gln Thr Gly Asn Glu Tyr Lys Tyr Lys Glu Asn
305 310 315 320

Leu Ile Thr Phe Ile Asp Asn His Asp Met Ser Arg Phe Leu Ser Val
325 330 335

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Asn Ser Asn Lys Ala Asn Leu His Gln Ala Leu Ala Phe Ile Leu Thr
340 345 350

Ser Arg Gly Thr Pro Ser Ile Tyr Tyr Gly Thr Glu Gln Tyr Met Ala
355 360 365

Gly Gly Asn Asp Pro Tyr Asn Arg Gly Met Met Pro Ala Phe Asp Thr
370 375 380

Thr Thr Thr Ala Phe Lys Glu Val Ser Thr Leu Ala Gly Leu Arg Arg
385 390 395 400

Asn Asn Ala Ala Ile Gln Tyr Gly Thr Thr Thr Gln Arg Trp Ile Asn
405 410 415

Asn Asp Val Tyr Ile Tyr Glu Arg Lys Phe Phe Asn Asp Val Val Leu
420 425 430

Val Ala Ile Asn Arg Asn Thr Gln Ser Ser Tyr Ser Ile Ser Gly Leu
435 440 445

Gln Thr Ala Leu Pro Asn Gly Ser Tyr Ala Asp Tyr Leu Ser Gly Leu
450 455 460

Leu Gly Gly Asn Gly Ile Ser Val Ser Asn Gly Ser Val Ala Ser Phe
465 470 475 480

Thr Leu Ala Pro Gly Ala Val Ser Val Trp Gln Tyr Ser Thr Ser Ala
485 490 495

Ser Ala Pro Gln Ile Gly Ser Val Ala Pro Asn Met Gly Ile Pro Gly
500 505 510

Asn Val Val Thr Ile Asp Gly Lys Gly Phe Gly Thr Thr Gln Gly Thr
515 520 525

Val Thr Phe Gly Gly Val Thr Ala Thr Val Lys Ser Trp Thr Ser Asn
530 535 540

Arg Ile Glu Val Tyr Val Pro Asn Met Ala Ala Gly Leu Thr Asp Val
545 550 555 560

Lys Val Thr Ala Gly Gly Val Ser Ser Asn Leu Tyr Ser Tyr Asn Ile
565 570 575

Leu Ser Gly Thr Gln Thr Ser Val Val Phe Thr Val Lys Ser Ala Pro
580 585 590

Pro Thr Asn Leu Gly Asp Lys Ile Tyr Leu Thr Gly Asn Ile Pro Glu
595 600 605

Leu Gly Asn Trp Ser Thr Asp Thr Ser Gly Ala Val Asn Asn Ala Gln
610 615 620

Gly Pro Leu Leu Ala Pro Asn Tyr Pro Asp Trp Phe Tyr Val Phe Ser
625 630 635 640

Val Pro Ala Gly Lys Thr Ile Gln Phe Lys Phe Phe Ile Lys Arg Ala
645 650 655

Asp Gly Thr Ile Gln Trp Glu Asn Gly Ser Asn His Val Ala Thr Thr
660 665 670

Pro Thr Gly Ala Thr Gly Asn Ile Thr Val Thr Trp Gln Asn
675 680 685